TRANSGENIC ANIMALS AS URINARY BIOREACTORS FOR THE PRODUCTION OF POLYPEPTIDE IN THE URINE, RECOMBINANT DNA CONSTRUCT FOR KIDNEY-SPECIFIC EXPRESSION, AND METHOD OF USING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of application 09/438,785, filed November 12, 1999, which claims priority under 35 U.S.C. §119(e) from U.S. provisional application 60/108,195, filed November 13, 1998, and U.S. provisional application 60/142,925, filed July 9, 1999, the entire contents of each of these prior applications are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to transgenic animals as urinary bioreactors for the expression and production of polypeptides in the urine. The present invention further relates to a recombinant DNA construct for kidney-specific expression of polypeptides in the urine and to a method for producing such polypeptides in the urine.

Description of the Related Art

Significant progress has recently been made in using transgenic animals as bioreactors to produce large quantity and high quality pharmaceuticals. The overall strategy entails the use of tissue-specific promoters to drive the expression of genes encoding medically important molecules. When those molecules are expressed in the target tissue of

transgenic animals and secreted into body fluids, they can be harvested, purified and used for treating human diseases. The most notable example is the milk-based bioreactor system, taking advantage of mammary gland-specific gene promoters. U.S. Patent No. 5,476,995 was one of the first patents directed to transgenic female sheep as milk-based bioreactors that expressed the transgene in the mammary gland so as to produce the target protein in its milk.

A number of proteins have been produced in milk-based bioreactor systems, such as protein C (U.S. Patent No. 5,589,604), blood coagulation factors (U.S. Patent No. 5,322,775), fibrinogen (U.S. Patent No. 5,639,940), antibodies (U.S. Patent No. 5,625,126) and hemoglobin (U.S. Patent No. 5,602,306), some of which are now being used in clinical trials. However, even in view of its initial success, a milk-based bioreactor system has several limitations. The first relates to its relatively low degree of cost-effectiveness. For instance, the lactation of transgenic livestock does not occur until an average of one and a half years old. Besides, lactation only occurs in female animals and lasts for a limited period of time. Secondly, purification of target proteins from milk often requires the development of complicated purification schemes (Wilkins et al, 1992). Thirdly, leakage of biologically active proteins from the mammary gland into the blood stream commonly occurs with the possibility of leading to pathological conditions in transgenic animals.

Another potential bioreactor system that can circumvent some of the above-mentioned limitations is a urine-based system where urine is an easily collectable fluid from transgenic livestock animals. This bioreactor system has been recently tested by Kerr and colleagues (1998), among whom is one of the present inventors, in transgenic mice using a urothelium-specific promoter (uroplakin II promoter) to drive human growth hormone (hGH) expression and production. They found that hGH could indeed be found in the urine of these transgenic mice at a concentration of 0.1 mg/ml, indicating that the urothelium can serve as an alternate bioreactor. The major advantages of this urine-based system over milk-based systems are the ability to harvest the product soon after birth and throughout the life of the animal irrespective of sex or reproductive status and the ease of product purification from urine. In addition, livestock urine is a proven, currently utilized source of pharmaceuticals; it is estimated that urine is being collected from 75,000 pregnant horses annually as a source of estrogenic compounds for postmenopausal hormone replacement therapy (Williams, 1994).

Despite these major advantages, several technical problems still exist with the above-mentioned urine-based bioreactor system, the most important being the relatively low yield of urinary hGH (0.1 mg/ml) obtained by Kerr et al (1998), as most of the hGH appear to be trapped in the cytoplasm of the superficial urothelial cells. This relatively low yield may be because the urothelium is not known to be a major secretory epithelium and the purification

of a minor protein from urine may require sophisticated purification procedures. In addition, low levels of hGH was found to have leaked into the mouse blood stream, possibly being responsible for the infertility observed in the transgenic female mice.

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SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the above-mentioned deficiencies in the art by providing a urine-based bioreactor system using a kidney-specific promoter for the expression and production of a recombinant biologically active polypeptide and a targeting system for the apical surface membrane of kidney epithelial cells.

The present invention provides a recombinant DNA molecule containing a kidney-specific promoter operably linked to a heterologous DNA sequence, which kidney-specific promoter is capable of expressing the heterologous biologically active polypeptide, encoded by the heterologous DNA sequence and containing an apical membrane targeting system, in the kidney of a host animal to produce a recombinant biologically active polypeptide in the urine.

As an embodiment of the present invention, the heterologous biologically active polypeptide contains a glycosyl phosphatidylinositol (GPI) signal sequence at its C-terminus to target the heterologous biologically active polypeptide to the apical surface of kidney epithelial cells for secretion into the lumen. In another embodiment, the heterologous biologically active polypeptide can be expressed as a fusion polypeptide between a biologically active polypeptide of interest and uromodulin via a protease-sensitive linker. The C-terminus of this fusion polypeptide is the C-terminus of uromodulin and contains a GPI signal sequence.

A further embodiment of the present invention provides for introducing one or more non-native sites for glycosylation into the heterologous biologically active polypeptide.

Yet another embodiment of the present invention is directed to an operable linkage of the kidney-specific promoter to both the heterologous DNA sequence encoding a heterologous biologically active polypeptide and a DNA sequence encoding phosphatidylinositol-specific phospholipase C (PIPLC), which DNA sequence encoding PIPLC is positioned downstream from the heterologous DNA sequence relative to the kidney-specific promoter.

A further object of the present invention provides a urine-based bioreactor system in which apical surface membrane targeting is enhanced by the inactivation or deletion of

basolateral surface membrane targeting signals in the recombinant biologically active polypeptide.

The present invention also provides for a method for producing a recombinant biologically active polypeptide *in vivo* using a urine-based bioreactor system in transgenic animals. Further provided are transgenic animals, all of whose somatic cells and preferably all of whose germ cells contain a recombinant construct or transgene from which a biologically active polypeptide is produced in recoverable amounts in the urine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the apical and basolateral surfaces of kidney epithelial cells in relation to the urine space (lumen) and blood vessels.

Figure 2 is a schematic diagram showing an embodiment of a GPI-containing construct. The construct contains, from 5' to 3', the uromodulin promoter, hGH gene, and an in-frame GPI signal sequence followed by a stop codon and polyadenylation signal.

Figure 3 is an amino acid sequence comparison/alignment of rat (SEQ ID NO:38), mouse (SEQ ID NO:39), human (SEQ ID NO:40), and bovine (SEQ ID NO:41) uromodulin. Boxes represent potential Asn-linked glycosylation sites and underlines represent the GPI attachment site and indicate that the sequence in this GPI attachment site of uromodulin is highly conserved across species.

Figure 4 shows a restriction digestion of five phage clones (lanes 1-5) on agarose gel electrophoresis. M represents lanes of molecular weight markers.

Figure 5 shows a Southern blot corresponding to the agarose gel shown in Fig. 4 hybridized separately with each of the 5'-end, middle region, and 3'-end probes.

Figure 6 shows an agarose gel electrophoresis of PCR reaction products using the sets of primers for the 5'-end, the middle region, and the 3'-end of the uromodulin gene.

Figures 7A and 7B show agarose gel electrophoresis (Fig. 7A) of EcoRI restriction digests of genomic DNA from various animal species and Southern blot hybridization (Fig. 7B) of the restriction digested genomic DNA with the middle region probe.

Figure 8 is a schematic representation of the uromodulin (THP) gene structure in the human, bovine and rat genome. The open boxes represent exons with the exon numbering provided, and the thick bars represent the introns, the lengths of which are variable.

Figure 9 shows Southern blot hybridization of BAC plasmid clone 1 digested with the restriction enzymes, PstI (lane 4), ApaI (lane 6), EcoRI (lane 7), SacI (lane 8), and KpnI (lane 10) and hybridized separately with 5'-end, middle region and 3'-end probes.

Figures 10A-10H show the nucleotide sequence of the mouse uromodulin promoter region (SEQ ID NO:1) which is 9,345 bp upstream of the first mouse uromodulin coding exon.

Figure 11 is a schematic presentation of the mouse uromodulin promoter in which the arrow denotes the transcription initiation site, the letters denote restriction sites (A, ApaI; P, PstI; B, BamHI; H, HindIII; S, SpeI), and the short bar denotes the relative size of the DNA.

Figure 12 shows the partial cDNA sequence of goat uromodulin gene (SEQ ID NO:2). The location of primers AS14, AS15 and AS17 used for isolation of goat uromodulin genomic DNA is shown in underline.

Figure 13A and 13B show the nucleotide sequence of goat uromodulin gene intron 1 (Fig. 13A; SEQ ID NO:3) and exon 3 (Fig. 13B, SEQ ID NO:4). The location of primers AS1, AS2, AS3, AS4 and AS5 used in genomic walking is indicated.

Figures 14A and 14B show the nucleotide sequence of the goat uromodulin promoter region (SEQ ID NO:37). The boxed sequence denotes the TATA box and the arrow denotes the putative transcription initiation start site.

Figures 15A and 15B show a homology comparison of goat and mouse uromodulin promoter regions corresponding to nucleotide positions 1121-1629 in SEQ ID NO:37 and nucleotide positions 6679-7191 in SEQ ID NO:1 (designated in Figs. 15A and 15B as nucleotides 6677-7189), respectively. Gaps are denoted by a period (.) between nucleotides.

Figure 16 is a schematic diagram illustrating the construction of chimeric gene with a mouse uromodulin promoter and the coding sequence of human growth hormone. A 3.0 kb 5'-upstream sequence of the mouse uromodulin gene was cloned upstream of a 2.1 kb human growth hormone coding sequence.

Figure 17 shows a Southern blot analysis of mouse tail DNA of founder mice. Lanes 1 and 5 are non-transgenic control mice showing the endogenous fragment (Endo) of uromodulin coding sequence that hybridized with the uromodulin probe.

Figure 18 shows the results of a radioimmunoassay in the detection of hGH in the urine of transgenic mice.

Figure 19 shows a comparison of the urine and serum concentrations of hGH in transgenic mice.

Figure 20 shows a radioimmunoassay recovery test of hGH in test mice.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the development of a bioreactor system in a transgenic mammal where a recombinant biologically active polypeptide is produced and secreted into the urine by the kidney-specific expression of a heterologous polypeptide, which is encoded by a heterologous DNA sequence, under the direction of a kidney-specific promoter, such as the uromodulin promoter. This urine-based mammalian bioreactor system, according to the present invention, is obtained by producing a transgenic mammal in which an isolated DNA molecule containing a recombinant construct or "transgene" for kidney- specific expression and production of the biologically active protein of interest is stably introduced. An example of a urine-based bioreactor system where the protein of interest is expressed in urothelial cells, rather than kidney cells, but which serves as guidance to development of a urine-

based bioreactor system, is provided by Lin et al (1995) and Kerr et al (1998). The present invention advantageously combines kidney-specific expression with apical surface membrane targeting to overcome the problems associated with leakage of an expressed heterologous biologically active polypeptide into the bloodstream.

To produce transgenic animals, any method known in the art for introducing a recombinant construct or transgene into an embryo, such as microinjection, cell gun, transfection, liposome fusion, electroporation, and the like, may be used. However, the most widely used method for producing transgenic animals, and the method most preferred according to the present invention, is microinjection, which involves injecting a DNA molecule into the male pronucleus of fertilized eggs (Brinster et al, 1981; Costantini et al, 1981; Harbers et al, 1981; Wagner et al, 1981; Gordon et al, 1976; Stewart et al, 1982; Palmiter et al, 1983; Hogan et al, 1986; U.S. Patent No. 4,870,009; U.S. Patent No. 5,550,316; U.S. Patent No. 4,736,866; U.S. Patent No. 4,873,191). While the above methods for introducing a recombinant construct/transgene into mammals and their germ cells were originally developed in the mouse, they were subsequently adopted for use with larger animals, including livestock species (WO 88/00239, WO 90/05188, WO 92/11757; and Simon et al, 1988). Microinjection of DNA into the cytoplasm of a zygote can also be used to produce transgenic animals.

Alternatively, a recombinant construct or transgene can be introduced into embryonic stem cells (ES cells) by any

method known in the art, such as those identified above as non-limiting examples. The ES cells transformed with the transgene are combined with blastocyst of the same animal species to colonize the embryo (Jaenisch, 1988). In some embryos, these transformed ES cells form the germline of the transgenic animal generated by this procedure. Transformed ES cells can also be used as a source of nuclei for transplantation into an enucleated fertilized oocyte to produce a transgenic animal.

The present invention for producing a biologically active polypeptide in a urine-based mammalian bioreactor system is not limited to any one species of animal, but provides for any appropriate non-human mammal species. For example, while mouse is a mammal species that is routinely used for producing transgenic animals and, thus, serves as a model system to test the transgene, other non-limiting but preferred examples include farm animals, such as pigs, sheep, goats, horses and cattle, which generate large quantities of urine, may be suitably used. A most preferred animal for use as a urinary bioreactor is a goat.

The success rate for producing transgenic animals by microinjection is highest in mice, where approximately 25% of fertilized mouse eggs into which the DNA has been injected, and which have been implanted in a female, will develop into transgenic mice. Although a lower success rate has been achieved with rabbits, pigs, sheep and cattle (Jaenisch, 1988; Hammer et al, 1985 and 1986; Wagner et al, 1984), the production of transgenic livestock is considered by those in

the art to be routine and without undue experimentation. Wall et al (1997a), Velander et al (1997), Drohan (1997), Hyttinen et al (1994), Morcol et al (1994), Lubon et al (1997), Houdebine (1997), Wall et al (1997b), Van Cott et al (1997), Cameron (1997), Cameron et al (1994), Niemann (1998) and Hennighausen (1992), among others, have reported and discussed the use of livestock as bioreactors or factories for the production of biologically active proteins.

The introduction of a DNA containing a transgene sequence at the fertilized oocyte stage ensures that the introduced transgene will be present in all of the germ cells and somatic cells of the transgenic animal. The presence of the introduced transgene in the germ cells of the transgenic "founder" animal, in turn, means that all of the founder animal's offspring will carry the introduced transgene in all of their germ cells and somatic cells.

There is no need for incorporating any plasmid or viral sequences with the gene being introduced, (Jaenisch, 1988), although the vector sequence may be useful in some instances. In many cases however, the presence of vector DNA has been found to be undesirable (Hammer et al, 1987; Chaka et al, 1985 and 1986; Kollias et al, 1986; Shani 1986; Townes et al, 1985). For instance, the transgene construct can be excised from the vector used to amplify the transgene in a microbial host by digestion with appropriate restriction enzymes. The transgene is then recovered by conventional methods, such as electroelution followed by phenol extraction and ethanol precipitation, sucrose density gradient

centrifugation, chromatography, HPLC, or combinations thereof. It has been reported in U.S. Patent No. 5,589,604 that high transformation frequencies, on the order of 20% or more, in both mice and pigs were obtained by microinjection with HPLC-purified DNA.

In order for the introduced gene sequence to be capable of being specifically expressed in the kidney of the transgenic animal, the gene sequence must be operably linked to a kidney-specific promoter. A DNA molecule is said to be "capable of expressing" or "capable of directing the expression of" a polypeptide if it contains nucleotide sequences which contain cis-acting transcriptional regulatory information, and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The cis-acting regulatory regions needed for gene expression in general include a promoter region, and such regions will normally include those 5'-non-coding sequences involved with initiation of transcription. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, the DNA sequence encoding a polypeptide of interest is operably linked to a kidney-specific promoter to generate a recombinant construct or "transgene" that is then introduced into the fertilized embryo or ES cells.

Also included in the transgene are nucleotide sequences that encode the signal sequences that direct secretion of the expressed biologically active polypeptide of interest into the urine of the transgenic animal. Both endogenous and heterologous signal sequences (either for the host or for the biologically active protein of interest) can be used, although the endogenous signal sequence of the heterologous protein of interest is preferred. Furthermore, other regulatory sequences in addition to the promoter, such as enhancers, splice signals, ribosome binding sites and polyadenylation sites, etc., may be useful in the transgene construct as would be well-recognized by those of skill in the art.

The preferred promoter in the recombinant construct/ transgene for the kidney-specific expression of a heterologous biologically active polypeptide of interest is the promoter for uromodulin. Uromodulin, also named Tamm-Horsfall protein (THP), is by far the most abundant urinary protein of human and other higher mammals, with an excretion rate of up to 200 mg per day (Hunt et al, 1985; Reinhart et al, 1989). This ~90 kDa glycoprotein has several important features that are relevant to its use in a kidney-expressed urine-based bioreactor system. The protein is synthesized by the epithelial cells of the ascending limb of Henle's loop and the beginning portion of the distal convoluted tubule, delivered exclusively to apical membrane and secreted into the urine (Sikri et al, 1981; Bachmann et al, 1990). Rindler et al (1990) established that uromodulin is a cell surface protein

anchored onto the apical plasma membrane via a glycosylphosphatidyl inositol (GPI) tail, where phosphatidylimositol-specific phospholipase C (PIPLC) cleavage *in vitro* of the GPI linkage completely releases the molecule into the culture medium.

Uromodulin is highly tissue-specific, being expressed only in the kidneys and not in any other epithelial and mesenchymal tissue. Moreover, uromodulin is evolutionarily conserved throughout placental animals. The cDNA sequences reported for rat uromodulin (Fukuoka et al, 1992) and human uromodulin (Hession et al, 1987; Pennica et al, 1987) were found to be 91% and 77% identical with the mouse uromodulin cDNA sequence, respectively (Prasadan et al, 1995). Prasadan and colleagues (1995) also reported that an alignment of uromodulin amino acid sequences from mouse, rat and human showed 91% similarity and 86% identity between mouse and rat, and 79% similarity and 70% identity between mouse and man.

As discussed in the Example 1 presented herein, the laboratory of the present inventors has isolated and sequenced a 9,345 base pair region including about 7 Kb upstream of the coding region of the mouse uromodulin gene, which region contains the mouse uromodulin promoter. This DNA promoter region, or a fragment thereof which retains the tissue specific promoter activity thereof, is used for construction of a transgene with a biologically active polypeptide of interest, i.e., human growth hormone (hGF). While knowledge of the nucleotide sequence of the mouse uromodulin promoter

would facilitate the construction of a transgene which is capable of kidney-specific expression of a biologically active polypeptide of interest, such sequence information is not necessary because it is well within the skill of the art to isolate a functional promoter sequence given a uromodulin genomic clone with the upstream promoter region. There is a wealth of scientific literature directed to the isolation and identification of a promoter for a given gene, with the Kahari et al (1990) article on the delineation of functional promoter and regulatory cis-elements being just one representative citation. Clones containing the goat uromodulin gene promoter have also been obtained as disclosed herein in Example 2 with the sequence of the goat uromodulin promoter being presented in Figs. 11A and 11B. Other uromodulin gene promoters can be further isolated using the genomic walking procedure described for the isolation of the mouse and goat uromodulin gene promoters in the Examples herein.

As a preferred embodiment of the present invention, a uromodulin-based urine bioreactor system has the following advantageous features:

(1) Uromodulin is a kidney-specific and abundantly expressed gene and its synthesis is confined to the thick-ascending limb of Henle's loop and early distal tubules of the kidneys. Biologically important genes under the control of uromodulin promoter are likely to be expressed in the same location and secreted into the urine, where the expressed gene products can be readily purified.

(2) Year-round production, which is independent of age and sex as compared to mammary-based bioreactor.

Uromodulin has already been reported to be evolutionarily conserved, being detectable immunologically in all placental mammals (Kumar et al, 1990). The laboratory of the present inventors has shown by Southern blot hybridization that the uromodulin gene is present as a single copy in many mammals, including all important livestock, such as cattle, sheep, goat, horse and pig. Not only do the uromodulin cDNAs from human, mouse and rat share a high level of identity (on the order of 80% or more), but even the high mannose glycosylation of uromodulin is highly conserved among different species of mammals. This strongly suggests that the promoter sequences of uromodulin are also likely to be conserved among mammals.

Moreover, as evidenced by the numerous examples in the scientific literature of promoters that are interchangeable among species, the uromodulin promoter from one mammal species is believed to be functional in another species. Accordingly, the mouse uromodulin promoter identified herein may be able to be used directly in transgenic livestock to drive kidney-specific expression of the biologically active polypeptide of interest in a urine-based bioreactor system. Alternatively, the uromodulin promoter used in the transgenic livestock to drive kidney-specific expression of the biologically active polypeptide can be its own endogenous uromodulin promoter, such as using the goat uromodulin to drive kidney-specific expression in

transgenic goats, or an interchangeable uromodulin promoter from another species of livestock. A computer comparison of the nucleotide sequences of the goat and mouse uromodulin promoter regions determined by the laboratory of the present inventors only found homology (approximately 74%) over a short stretch of about 500 bp that includes the first exon of uromodulin (Fig. 12A). No other significant homology was found within about 1,100 bp of the promoter region 5'-upstream of this short stretch of homology. If it is later determined that the mouse promoter or any other non-native uromodulin promoter does not provide sufficiently kidney-specific expression in the transgenic animal, then the native uromodulin promoter would be used instead in the transgene construct.

The bovine and rat uromodulin promoter regions have already been identified in Yu et al (1994), the entire contents of which are hereby incorporated herein by reference. Specifically, Fig. 5 of Yu et al (1994) shows the nucleotide sequence of the bovine and rat uromodulin promoter regions. These promoter regions, or a fragment thereof with kidney-specific promoting activity, can be used to drive the kidney-specific expression of a heterologous gene in those respective species. If it is determined that the regions of the approximately 600 base pairs upstream of the transcription start site in the bovine and rat sequences of Fig. 5 of Yu et al (1994) do not contain the complete kidney-specific uromodulin promoter sequence for these species, additional nucleotides upstream of the disclosed sequences can readily be

obtained and sequenced using the specific sequences as a probe of bovine and rat genomic libraries, or using the technique of genomic walking as described in the examples herein, without the use of undue experimentation.

Uromodulin promoters from other mammalian species can be isolated using the same approaches outlined in the examples provided herein, or using the same approach used in Yu et al (1994), or by hybridization or PCR amplification of genomic libraries or genomic DNAs using probes or primers from the genomic clones of the mouse, goat, rat or cow uromodulin gene. If the need to use a uromodulin promoter from another livestock animal species arises, then information generated from the mouse and goat uromodulin promoters or from the bovine and rat uromodulin promoter region of Yu et al (1994) can be used to facilitate this process. For instance, as the sequence of the mouse and goat uromodulin promoters have now been determined and are reported herein, and the bovine, rat and human promoter regions have been previously reported, oligonucleotide primers based on these sequences can be designed for PCR reactions. Long-range PCR can be performed to directly isolate uromodulin promoters from a pool of genomic DNAs extracted from various livestock animal species. DNA fragments containing the uromodulin promoter from livestock animal species can also be identified by hybridization of genomic libraries of corresponding species with mouse, goat, bovine, rat or human uromodulin promoter probes under hybridization conditions similar to or the same

as that used for the Southern blots (Zoo-blots of genomic DNA from various species) disclosed in Example 1 provided herein.

As will be appreciated by those in the art, the uromodulin promoter or any other kidney-specific promoter used in the transgene for directing kidney-specific expression of the biologically active polypeptide of interest can include relatively minor modifications, such as point mutations, small deletions or chemical modifications that do not substantially lower the strength of the promoter or its tissue-specificity.

In addition, the identification of additional promoters active in directing gene expression in the kidney can be routinely performed using the suppression subtraction hybridization library technique. Using this technique, which eliminates the cDNAs that are shared by multiple tissues (Diatchenko et al, 1996), a library highly enriched in kidney-specific cDNAs can be generated. Total RNAs are isolated from stomach, intestine, colon, liver and brain, and Northern blot analysis of these mRNAs using an actin cDNA as a probe is used to demonstrate the intactness of the actin mRNA in all of these preparations. Kidney cDNAs are then used as the "tester", and the cDNAs of all the other non-kidney tissues, referred to as the "drivers", are subtracted from the kidney cDNAs. Using the subtraction library technique, the laboratory of the present inventors had earlier probed the cDNAs of the non-subtracted and the subtracted libraries with actin cDNA or uroplakin Ib cDNA, and the results indicated that the original (non-subtracted) bovine bladder cDNA

preparation contained abundant actin mRNA and relatively little uroplakin Ib mRNA. In contrast, the subtracted library contained almost no detectable actin mRNA (at least 50 fold reduction) but greatly increased uroplakin Ib mRNA (>10 to 15 fold enrichment). Multiple cDNA clones have been isolated from the subtraction library and used to probe the mRNAs of various bovine tissues. For example, a uroplakin Ib probe confirmed its bladder specificity.

The laboratory of the present inventors have already been successful in obtaining three unidentified cDNAs in which the tissue distribution pattern showed bladder specificity. Sequencing data indicate that these three bladder-specific clones are novel genes not described previously. In the same manner, kidney-specific genes can be isolated, and any gene that is involved in the structure and function of the excretory tract of the kidney, including proximal, distal tubules, Henle's loop, collecting duct system can be applied in this system to isolate its promoter for use in expressing and producing a biologically active protein in a urine-based kidney bioreactor. Although the suppression subtraction hybridization library technique is the preferred procedure for obtaining tissue-specific genes, kidney-specific genes can also be identified through other well-known methods, including biochemical methods, protein chemistry, monoclonal antibody production, two-dimensional gel electrophoresis, cDNA library screening, expression library screening, differential display, phage display, etc.

Although there is an abundance of evidence suggesting that many important regulatory elements are located 5' to the mRNA cap site (McKnight et al., 1982; Payvar et al., 1983; Renkowitz et al., 1984; Karin et al., 1984) and in a great majority of cases the 5'-flanking region is sufficient to convey the tissue-specificity and high-level expression of a tissue-specific gene, it has been reported that in some instances important regulatory elements, particularly those mediating tissue-specific expression, may reside within the structural gene, i.e., introns, or even the 3'- to it in the untranslated sequences, and contribute to promoter activity (Charnay et al., 1984; Gillies et al., 1983; Sternberg et al., 1988). For example, intron I sequences were found to be important for high-level and tissue-specific expression of an α-skeletal actin gene, a β-globin gene and a peripherin gene (Reecy et al, 1998; James-Pederson et al, 1995; Belecky-Adams et al, 1993). In view of these examples of introns or 3'-untranslated sequences contributing to promoter activity, the constructs to be made may include intron I sequences of a kidney-specific gene and, when necessary, 3'-untranslated sequences placed downstream of the DNA sequence encoding the heterologous polypeptide of interest according to the present invention. In the former case, a fragment will be isolated that spans the 5'-flanking region, the first exon and the first intron, followed by the DNA sequence encoding the biologically active polypeptide of interest. The translation initiation codon of the kidney-specific gene could also be mutated to avoid translation of a truncated protein, and other

regions of the kidney-specific gene could also be used to ensure the tissue-specific and high-level expression of the transgene.

As used herein, "biologically active polypeptide" refers to a polypeptide/protein capable of causing some effect within an animal and preferably not within the animal having the transgene. Examples of such polypeptides/proteins include, but are not limited to, adipokinin, adrenocorticotropin, blood clotting factors, chorionic gonadotropin, corticoliberin, corticotropin, cystic fibrosis transmembrane conductance regulators, erythropoietin, folliberin, follitropin, glucagon gonadoliberin, gonadotropin, human growth hormone, hypophysiotropic hormone, insulin, lipotropin, luteinizing hormone-releasing hormone, luteotropin, melanotropin, parathormone, parotin, prolactin, prolactoliberin, prolactostatin, somatoliberin, somatotropin, thyrotropin, tissue-type plasminogen activator, vasopressin, antibodies, peptides, and antigens (for use in vaccines). It will be appreciated by those of skill in the art that the above list is not exhaustive. In addition, new genes for biologically active proteins that will function in the context of the present invention are continually being identified.

Proteins which degrade or detoxify organic material may also be produced by means of the present invention. Such proteins may be those discussed in WO 99/28463, the entire contents of which is hereby incorporated by reference.

The biologically active polypeptide produced in the urine-based bioreactor system according to the present

invention can be isolated from the urine of these transgenic animals. Accordingly, the present invention provides a means for isolating large amounts of biologically active polypeptides from the urine of transgenic animals which can be used for a variety of different purposes. Furthermore, the biologically active polypeptide can be readily recovered and purified from the urine as would be well within the skill of those in the art.

Because the uromodulin promoter is a preferred promoter for the kidney-based urinary bioreactor system according to the present invention, a transgenic mouse model, in which a mouse uromodulin promoter is operably linked to a DNA sequence encoding human growth hormone, was generated. As described in Example 3, a transgene containing a 3.0 kb mouse uromodulin promoter and 2.1 kb human growth hormone gene was constructed and injected into the fertilized eggs of FVB/N inbred mice. Out of the 42 live-born animals, three animals carried the transgene as evidenced by the appearance of a 5.1 kb transgene fragment in Southern blot hybridization of tail DNA. Upon radioimmunoassay, two of these founder mice were found to secret human growth hormone into the urine. Unexpectedly however, one of the two positive mice that secreted the human growth hormone died at 4 months of age. The remaining positive mouse showed, in addition to urinary hGH, a high concentration of hGH in the serum. These observations, together with the result that the remaining positive male mouse failed to impregnate two batches of female mates strongly indicate that the leakage of hGH into the serum

inadvertently affected the physiology and reproductive ability of the founder animals.

Although the adverse effects of leakage of biologically active molecules into the bloodstream have been well documented in the transgenic bioreactor field, definite solutions are scarce, if not nonexistent. The leakage into the bloodstream in transgenic animals can result in severe consequence including the loss of capacity of the bioreactor, rendering it inefficient or inoperable. In the case of urine-based bioreactor, the yield of hGH is compromised; the leakage of hGH into the bloodstream leads to premature death and infertility of the animals. The success of this bioreactor system therefore largely depends upon whether the leakage problem can be solved.

Cell membranes in polarized epithelial cells are functionally divided into apical and basolateral membranes (FIG.1). The problem of leakage of hGH into the bloodstream is due to the non-directed secretion of hGH into both the apical surface and the basolateral sides of the membrane which are in close vicinity to blood vessels underlying the epithelial cell layer. A unique aspect of the present invention is directed to apical membrane targeting and urinary secretion of the recombinant proteins, which apical targeting minimizes basolateral leakage of the biologically active polypeptide of interest into the bloodstream and thereby also increasing the amount of hGH being secreted into the urine. When a recombinant polypeptide is targeted to the basolateral surface or lacks an apical targeting signal, this protein can be

easily leaked into the blood, potentially causing pathological conditions in transgenic animals. Enhanced apical targeting in uromodulin-synthesizing cells will overcome this problem because the recombinant polypeptide will be directly released into the urinary space.

While a great majority of cellular proteins are either secreted or permanently anchored onto the cell membrane, a small group of proteins are temporarily anchored onto the external surface of the plasma membrane via glycolipids. These anchors are termed glycosyl phosphatidylinositols (GPIs) and cleavage of the GPI by phospholipases can release the protein from the membrane. Although the exact function of the GPI linkage is unclear, one of the proposed functions for a GPI sequence is the possibility that GPI serves as an apical targeting signal. A GPI signal sequence usually contains two parts: a stretch of 17-30 hydrophobic amino acids at the very end of the C-terminus of a protein, which will be cleaved and thus be absent in mature proteins, and a shorter stretch (about 8-14 amino acids) containing small amino acids and serving as the GPI anchorage site. GPI structure and the biosynthesis of GPI anchored membrane proteins are reviewed in Englund (1993) and Udenfriend et al. (1995).

According to a preferred embodiment of the present invention, apical surface membrane targeting is provided by a GPI signal sequence. Therefore, in the present invention, a kidney-specific promoter, preferably the uromodulin promoter, drives the expression of a gene or cDNA encoding a recombinant

polypeptide with a GPI signal sequence placed at its C-terminus. This transgene construct will be achieved by constructing from 5'-end to 3'-end, a uromodulin promoter, a DNA sequence encoding a recombinant polypeptide, and a DNA sequence encoding a GPI signal (Fig. 2). This will allow the production of a recombinant polypeptide whose C-terminus is modified with a GPI signal sequence which will be linked with GPI. The GPI sequence can also be located at the N-terminus of the recombinant polypeptide or in the middle of a protein or a fusion protein. With the GPI as an apical targeting signal, the heterologous polypeptide is directed exclusively to the apical surface, instead of to both the apical and basolateral surfaces, where the heterologous polypeptide anchored to the apical membrane will be released into the urine by the action of PIPLC enzyme.

Figure 3 shows an amino acid alignment/comparison of rat, mouse, human, and bovine uromodulin. At the C-terminus, GPI signal sequence of rat (SEQ ID NO:42), mouse (SEQ ID NO:43), human (SEQ ID NO:44) and bovine (SEQ ID NO:45) are aligned and compared. The underlined sequences denote the GPI attachment site with the GPI addition site most likely being serine. It is clear that there is cross-species conservation of the GPI signal sequences between rat, mouse, human and bovine uromodulin.

Although the GPI signal sequence of uromodulin (THP) is preferred in the transgene construct according to the present invention because uromodulin is naturally targeted to the apical surface and because the uromodulin GPI signal

sequence is known to be efficiently cleaved *in vivo*, GPI signal sequences of other proteins, such as *Torpedo* acetylcholinesterase (Sikorav et al., 1988; SEQ ID NO:46), placenta alkaline phosphatase (Micanovic et al., 1988; SEQ ID NO:48), *T. brucei* PARP (Clayton et al., 1989; SEQ ID NO:49), hamster prion protein (Stahl et al., 1990; SEQ ID NO:50), rat Thyl (Seki et al., 1985; SEQ ID NO:51), *T. brucei* VSG (Boothroyd et al., 1980; SEQ ID NO:52), etc., can be suitably used. In the above-mentioned GPI signal sequences, GPI anchor addition involves the removal of residues C-terminal to residue 13 of SEQ ID Nos: 46-52. It should be noted that even though the GPI signal sequences of these other GPI anchored proteins are not highly sequence conserved, they have structural features that suffice for attachment of the GPI anchor.

An outline of a method for constructing a chimeric polypeptide containing a heterologous polypeptide of interest and GPI signal sequence at its C-terminus is as follows:

1) Creation of a restriction cloning site before the stop codon of hGH by site-directed mutagenesis using the vector containing the uromodulin-hGH construct described in Example 3.

2) Generation of cDNA fragment encoding a GPI-consensus sequence, preferably using the GPI signal sequence of uromodulin. PCR will be performed to amplify a DNA fragment encoding a GPI signal sequence. A restriction cloning site that is identical to the site before the stop codon within hGH will be incorporated into the PCR primers to facilitate

cloning, with caution being exercised to ensure that the GPI signal sequence is in the correct translational reading frame with the hGH sequence.

3) Cloning of the DNA fragment encoding the GPI signal sequence into the hGH-encoding DNA sequence.

4) Generation of transgenic mice producing hGH in the urine.

Based on a suitable GPI signal sequence, a universal GPI cassette that is applicable for cloning of a GPI signal sequence at the C-terminus of most, if not all, biologically active polypeptides can be constructed.

An alternative strategy for enhancing the apical secretion of recombinant polypeptides in urine-based kidney bioreactor is to produce a fusion protein between a desired polypeptide and uromodulin. This can be accomplished by constructing a DNA sequence containing the cDNA or gene encoding the desired polypeptide followed by a chemically or enzymatically cleavable linker sequence such as a protease-sensitive linker sequence (e.g., thrombin-sensitive sequence) and by a uromodulin cDNA sequence. This approach has several major advantages. First, since the endogenous uromodulin is predominantly targeted to the apical surface membrane, uromodulin can serve as a carrier for bringing the recombinant heterologous polypeptide to the apical surface. Second, since uromodulin has a tendency to form large, stable aggregates in the urine, the fused polypeptide will likely be more stable in aggregates than as a soluble polypeptide. Third, the aggregated fused polypeptide can be readily purified by first

centrifuging the urine to obtain the aggregates, and then cleaving the away the uromodulin portion by using a protease such as thrombin.

The release of uromodulin from the apical membrane into the urine requires the action of the PIPLC enzyme which specifically cleaves the GPI linkage. Likewise, the release of the GPI-linked recombinant polypeptide or recombinant polypeptide-uromodulin fusion protein in uromodulin-synthesizing cells would require a similar mechanism. Although, at the luminal surface of uromodulin-synthesizing cells, there naturally exists functional PIPLC, the amount of the enzyme may not be sufficient to handle large amounts of recombinant polypeptides with a GPI signal sequence. In this respect, overexpression of PIPLC under the direction of a kidney-specific promoter, preferably a uromodulin promoter, will ensure a sufficient amount of PIPLC to efficiently release GPI-anchored recombinant polypeptides from the apical surface. To do this, two constructs, one encoding the recombinant heterologous polypeptide and the other encoding PIPLC, could be co-injected into fertilized eggs to produce an animal bi-transgenic for the recombinant heterologous polypeptide and PIPLC. More likely however, two separate types of transgenic animals instead of a bi-transgenic animal are generated, one of which expresses the recombinant heterologous polypeptide of interest and the other expresses PIPLC. Bi-transgenic animals can then be readily produced by cross-breeding the two separate types of transgenic animals.

Another embodiment of apical surface membrane targeting according to the present invention is to make use of glycosylation of polypeptides as an apical targeting signal. Asn-linked glycosylation has been thought to be a facilitator of apical targeting signal for soluble and membrane proteins in epithelial cells. Although the mechanism is unclear, it has been hypothesized that the glycosylation may serve to interact with lectin-like molecules that are strategically located along the pathway toward the apical surface membrane. By adding one or more non-native glycosylation consensus sequences to a polypeptide which otherwise does not contain a glycosylation site (such as human growth hormone), one could achieve glycosylation, and thereby enhance apical targeting of the polypeptide. The glycosylation consensus sequence is the three amino acid sequence, Asn-Xaa-Ser/Thr, where Xaa can be any amino acid with the exception of proline and aspartic acid. To minimize the number of the amino acid substitutions in a given sequence, a strategy can be employed to introduce a non-native glycosylation site at a sequence containing Asn-Xaa-Xaa (the second Xaa being any amino acid other than Ser/Thr) to Asn-Xaa-Ser/Thr. Alternatively, an original sequence containing Xaa-Xaa-Ser/Thr can be changed to Asn-Xaa-Ser/Thr. To maximize the likelihood of the site being glycosylated, the sites will be designed at β-turns in the structure of the polypeptide, where such non-native sites will have a greater chance of being glycosylated. Globally, the glycosylation consensus sequence can be located at the N- or C-terminus or in the middle of the polypeptide, provided that

the mutation of a single amino acid does not impair the original biological function of the polypeptide. For any of the above-mentioned strategies for introducing a glycosylation consensus sequence, any method of site-directed mutagenesis can be performed on cDNA or gene encoding the polypeptide. In order to change a codon encoding any amino acid to Asn (AAU/C), a maximum of 3 point mutations, which can be easily accomplished by routine site-directed mutagenesis, would be required.

In addition to Asn-linked glycosylation, O-glycosylation has been shown to enhance the apical targeting of some epithelial membrane proteins. In general, the sites for O-glycosylation are clusters of serines and threonines (Sadeghi et al., 1999). Proline residues adjacent to serine and threonine residues enhances O-glycosylation (Yoshida et al; 1997). For example, the apical targeting of sucrase isomaltase, an intestinal brush border protein, requires the O-glycosylation of a stretch of 12 amino acids (Ala(37)-Pro (48)) juxtaposed to the membrane anchor. Yoshida et al. (1997) also reported that a sequence stretch containing Xaa-Thr-Pro-Xaa-Pro appears to be a good substrate for O-glycosylation. Accordingly, the Xaa-Thr-Pro-Xaa-Pro sequence stretch can also be introduced into the heterologous polypeptide of interest by site-directed mutagenesis.

An alternative strategy to produce a higher level of PIPLC than is normally produced in uromodulin-synthesizing kidney epithelial cells is to construct a DNA molecule in which a DNA sequence encoding PIPLC is placed 3'(downstream

from) of a construct where a kidney-specific promoter is operably linked to a DNA sequence encoding a heterologous polypeptide. The placement of the DNA sequence encoding PIPLC allows the kidney-specific promoter to be operably linked to both the DNA sequence encoding the heterologous protein and the DNA sequence encoding PIPLC. Thus, "bi-cistronic" mRNA can be transcribed from this particular type of construct.

An alternative to apical targeting by the addition of GPI or by glycosylation is the inactivation of potential basolateral targeting signals that are present in the heterologous polypeptide of interest. It has been reported that, in some instances, basolateral targeting depends on a distinctive cytoplasmic targeting signal, for example a tyrosine motif or a di-leucine motif.

The so-called tyrosine motif for basolateral targeting contains a consensus sequence YXXO where the first residue (Y) is tyrosine, the last amino acid (denoted by O) is a bulky hydrophobic amino acid residue (most commonly Leu), and the middle two residues can be any amino acid residue (Deschanbeault et al., 12991; Stephens et al., 1998). A double or di-leucine motif is also important for basolateral targeting. This motif is basically two (double) leucine residues (di-leucine; Hunziker et al., 1994). The tyrosine and di-leucine motifs are found frequently at the C-terminus of the protein or in the cytoplasmic domain of a membrane protein. Deletion or modification of these motifs will likely lead to the blockage of basolateral targeting. Experimental strategies to be employed in this alternative to apical

targeting include the removal of a segment at the C-terminus of a heterologous polypeptide that contains a basolateral targeting signal sequence, or the mutation of tyrosine and di-leucine motifs contained in basolateral targeting signal sequences on the heterologous polypeptide, such as by site-directed mutagenesis of the encoding DNA sequence.

As will be appreciated by those in the art, any combination of the aforementioned targeting approaches can be used. For example, the GPI and glycosylation approaches can be employed simultaneously, or the addition of GPI and/or glycosylation can be combined with the deletion/inactivation of basolateral targeting signal(s). Furthermore, these targeting approaches are not limited to targeting the apical surface membranes of kidney epithelial cells and are believed to also be applicable to other bioreacteor systems such as the mammary gland, urothelial, and seminal bioreactor systems.

While the production of transgenic animals by the introduction of the transgene into germ line cells is most preferred, it is also contemplated that the transgenic animals, which serve as a urinary bioreactor system, can be generated with vectors that are useful for transforming the kidney into a bioreactor capable of producing a biologically active protein in the urine for isolation. The transformed cells may be germ line or somatic cells.

In an alternative embodiment to introduction into germ line cells, the vector according to the present invention includes a system which is well received by the cells lining the excretory tract of the kidney, including proximal, distal

tubules, Henle's loop and collecting duct system. An example of a useful vector system is the Myogenic Vector System (Vector Therapeutics Inc., Houston, TX). In this embodiment, the heterologous DNA sequence encoding the biologically active polypeptide, linked to a viral promoter construct capable of directing kidney-specific expression and carried in the vector, is introduced into the kidney of an animal *in vivo*. Introduction of the vector can be carried out by a number of different methods routine to those of skill in the art. Vectors of the present invention can also be incorporated into liposomes and introduced into the animal in that form. The transgene is absorbed into one or more epithelial cells capable of expressing and secreting the biologically active protein into the urine collecting in the bladder.

Another alternative embodiment for generating a transgenic animal as a kidney-based bioreactor is through the use of targeted homologous recombination, where one copy of the endogenous uromodulin gene is disrupted by insertion of a heterologous gene encoding a biologically active molecule of interest, which heterologous gene is flanked by sequences complementary to the endogenous uromodulin gene. These flanking complementary sequences which direct homologous recombination to an endogenous uromodulin gene are at least 25 base pairs in length, preferably at least 150 base pairs. This technique for generating transgenic animals and cells by homologous recombination is disclosed in WO 90/11354 and U.S. Patent 5,272,071, the entire contents of which are hereby incorporated by reference. Accordingly, if it is desired for

the kidney to express and secrete a selected biologically active polypeptide into the urine, then a short sequence on either side of the start codon of the uromodulin coding sequence in a given species can be used as flanking sequences to create a construct that can be inserted at the specific location in the genome of the host animal species which is between the endogenous uromodulin gene promoter and the endogenous uromodulin gene coding sequence. In this way, the expression of the biologically active polypeptide of interest will be driven by the endogenous uromodulin promoter in the transgenic animal. The bovine genomic uromodulin sequence has already been reported (Yu et al., 1994), and the mouse genomic uromodulin sequence as well as the clone containing the goat genomic uromodulin gene sequence surrounding the start codon are disclosed herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1: ISOLATION OF MOUSE UROMODULIN GENE PROMOTER

Generation of Uromodulin cDNA Probes

Three probes corresponding to the 5'-end, the middle region and the 3'-end of the full-length uromodulin cDNA (Prasadan et al, 1995) were generated using the reverse transcription-polymerase chain reaction (RT-PCR) method, with three pairs of oligonucleotide primers chemically synthesized based on the published uromodulin cDNA sequence. The set of

primers for the 5'-end are 5'-TGGACCAGTCCTGTCCTGGTTCAG-3' (SEQ ID NO:5; sense), and 5'-GGGTGTTCACACAGCTGCTGTTGG-3' (SEQ ID NO:6; antisense). The set of primers for the middle region are 5'-AGGGCTTTACAGGGGATGGTTG-3' (SEQ ID NO:7) and 5'-GATTGCACTCAGGGGGCTCTGT-3' (SEQ ID NO:8) The set of primers for the 3'-end are 5'-GGAACTTCATAGATCAGACCCGTG-3' (SEQ ID NO:9) and 5'-TGCCACATTCCTTCAGGAGACAGG-3' (SEQ ID NO:10). These three pairs of oligonucleotide primers were used to amplify uromodulin cDNA fragments using, as a template, a pool of cDNAs reversed transcribed from mouse kidney RNAs. PCR conditions included the first cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; 35 cycles of 95°C for 2 min, 55°C for 1 min, and 72°C for 2 min; and the last cycle of 94°C for 2 min, 55°C for 1 min, and 72°C for 8 min. Agarose gel electrophoresis revealed a 400 bp, a 440 bp and a second 400 bp PCR product for the three sets of primer amplifications, 5'-end, middle region, and 3'-end, respectively. These PCR products were purified by extraction and chromatography using a QIAEX II method (QIAGEN, Valencia, CA).

Screening of Mouse Kidney cDNA Library

A mixture of the above three uromodulin cDNA probes were ^{32}P-labeled and used to screen a BALB/c mouse kidney cDNA library (Clontech, Palo Alto, CA). A total of 2 x 10^5 phage clones from the cDNA library were plated, lifted onto nylon membrane and hybridized with the mixture of probes at 42°C for 16 hours in a solution containing 50% Formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS and 100 mg/ml denatured salmon

sperm DNA. After hybridization, the nylon filters were washed at 65°C for 1 hour in 1X SSC and 0.1% SDS, and autoradiographed. Five phage clones were identified from the primary screening, and they were plaque-purified and subjected to the secondary screening using the same conditions as the primary screening. Purified phage clones were amplified by plate lysate and analyzed by EcoRI restriction digestion and agarose gel electrophoresis. On agarose gel, the five clones are of different sizes, ranging from 0.2 kb to 2.7 kb (Fig. 4). A 2.7 kb clone hybridized with all three probes indicating that this band likely represented the full-length mouse uromodulin cDNA clone (Fig. 5). This 2.7 kb band was excised from the bacteriophage with EcoRI restriction enzyme, gel-purified, subcloned into the same site of pBluescript KS^+ (Stratagene, LaJolla, CA), and sequenced. The sequence matched precisely with the published mouse (uromodulin cDNA sequence of Prasadan et al, 1995), further establishing the authenticity of this as mouse uromodulin.

Isolation of Mouse Uromodulin Gene

For the isolation of the mouse uromodulin gene, a commercial genomic screening service (Genomic System, St. Louis, MO) was used. Briefly, two pairs of PCR primers located in exon 3 (exon information derived from human uromodulin gene, Pennica et al, 1987) were designed and pre-tested by the present inventors. These primers were then used by Genomic System to mass-screen by PCR pooled genomic (BAC) plasmid clones of the MAC ES Mouse II library which harbors

129/SVJ mouse genomic DNAs. The first pair of primers, sense 5'- AGGGCTTTACAGGGGATGGTTG-3' (SEQ ID NO:11), and antisense 5'- GATTGCACTCAGGGGGCTCTGT-3' (SEQ ID NO:12), was used for the initial screen which yielded two uromodulin clones, each about 60-70 kb in length. These clones were confirmed independently by using a second set of nested primers, sense 5'-GCCTCAGGGCCCGGATGGAAAG-3' (SEQ ID NO:13) and antisense 5'-GCAGCAGTGGTCGCTCCAGTGT-3' (SEQ ID NO:14). In addition, PCR reactions using the three pairs of primers located at the 5'-end, the middle region and the 3'-end (SEQ ID NOs:5-10) showed that these two clones contained all the coding sequence information, indicating that it contained the entire uromodulin gene (Fig. 6).

Identification of the Uromodulin Gene in Multiple Animal Species

An analysis of the conservation of the uromodulin gene sequence in other animal species is shown in Figs. 7A and 7B. The genomic DNA of human, monkey, rat, mouse, dog, cow, rabbit, chicken and yeast were digested with EcoRI restriction enzyme and hybridized with the uromodulin middle region probe described above, using the same Southern blot hybridization conditions used above for screening the mouse kidney cDNA library. The results of the Southern blot hybridization shown in Fig. 7B show that the uromodulin gene is conserved in mammals and is present as a single copy in human, monkey, rat, mouse, dog, cow and rabbit. Pennica et al (1987) and Yu et al (1994) reported that the gene structure (exons and introns) of

human, bovine and rat uromodulin are highly conserved (Fig. 8).

Identification of Gene Fragments Containing the Mouse Uromodulin Gene Promoter

Southern blotting was performed to identify DNA fragments containing the uromodulin promoter sequence. This approach is based on the differential reactivity of DNA restriction fragments of BAC clone 1 DNA with three different uromodulin probes located in the 5'-end, middle region, and 3'-end of the uromodulin cDNA. Thus, BAC plasmid clone 1 was digested with the restriction enzymes NotI, BamHI, HindIII, PstI, EcoRI, ApaI, NcoI, SacI, XhoI and KpnI. After agarose gel electrophoresis, DNA fragments were transferred onto nylon membrane, UV-crosslinked and hybridized with the 5'-end, middle region, and 3'-end cDNA probes. A 6.9 kb PstI DNA fragment (Fig. 9, lane 4), an 8.3 kb ApaI DNA fragment (Fig. 9, lane 6), and an 8.5 kb SacI DNA fragment (Fig. 9, lane 8) reacted with only the 5'-end probe, but not with middle region probe or the 3'-end probe. This strongly indicates that these three DNA fragments contain portions of the 5'-end of the uromodulin coding sequence and, more importantly, a large fragment of the 5'-upstream region of the mouse uromodulin gene. In contrast, a 9 kb KpnI fragment reacted with all three probes (Fig. 9, lane 10), indicating that this fragment contains all the coding sequences for mouse uromodulin. Finally, a 10 kb EcoRI fragment reacted only with the 3'-probe (Fig. 9, lane 7), indicating that this fragment contains the

3'-end of the coding region and the non-coding region. The identification of DNA fragments containing the entire mouse uromodulin gene, particularly the 5'- upstream sequence facilitates the cloning of the uromodulin gene promoter.

Sequencing of Mouse Uromodulin Promoter

The 8.3 kb ApaI DNA fragment was used for further promoter analysis. A genomic walking method was employed to sequence the entire mouse uromodulin promoter from both 5'- and 3'-ends by sequentially walking the sequence and synthesizing the new primers based on newly obtained sequences. Sequences were determined by the dideoxynucleotide chain termination method of Sanger et al (1977) on an automatic DNA sequencer. Listed below are sense- and anti-sense primers used for the sequencing purposes.

Sense Primers

S1: 5'-TGTCCTATGTGACTCCAGCT-3' (SEQ ID NO:15)

S2: 5'-TCTCCTCAGCTCTCCTGGTC-3' (SEQ ID NO:16)

S3: 5'-TCCTGCCACCACCATGACCA-3' (SEQ ID NO:17)

S4: 5'-AAGCACCGGTGTGCTTGTAT-3' (SEQ ID NO:18)

S5: 5'-ATGGGGCTGCTGAGACTAAG-3' (SEQ ID NO:19)

Anti-sense Primers

AS1: 5'-AAGTCAGACTGTGTTAGGAT-3' (SEQ ID NO:20)

AS2: 5'-ATTGACTGAGCAGGAAGCAT-3' (SEQ ID NO:21)

AS3: 5'-ATTTTATAACCTCCCTCTAG-3' (SEQ ID NO:22)

AS4: 5'-ATGCATTCCAGTCTCAGTGC-3' (SEQ ID NO:23)

AS5: 5'-TGGGGAGAGGACAAAGCCTTG-3' (SEQ ID NO:24)

AS6: 5'-TGACGTGCCAACTCCACTGA-3' (SEQ ID NO:25)

AS7: 5'-AGGACCTGTAGGGTAAGAAA-3' (SEQ ID NO:26)

AS8: 5'-TCTGGCTGTGGGCTCTATAT-3' (SEQ ID NO:27)

Analysis of the Mouse Uromodulin Promoter

The 9,345 bp nucleotide sequence of the promoter region and the genomic coding region including exon 3 of the mouse uromodulin gene is shown in Fig. 10. These results (1) establish the authenticity of the isolated uromodulin clone, (2) indicate that a 7 kb uromodulin promoter has been obtained which is more than adequate to be used in the urine-based transgenic bioreactor system. This mouse promoter can be used in other mammalian species, such as farm animals, to drive the kidney-specific expression of any heterologous gene.

Subcloning of Mouse Uromodulin Promoter

Having identified the mouse uromodulin promoter region, this region can be subcloned for further amplification, and for constructing transgenes. Since the clone containing the uromodulin promoter region is at least 70 kb in size, restriction digestion of each of this clone gives rise to multiple bands. Although the relative sizes of uromodulin promoter-containing bands can be determined by Southern blotting using the 5'-end probe, this does not allow for pinpointing a specific band for subcloning, as most bands are not well-resolved. To circumvent this problem, a dot-blot approach by gel-purifying each individual band in the close vicinity of the area where Southern blot hybridization revealed a positive band will be taken. DNA in each band will

be eluted using a QIAEX column (QIAGEN), and then blotted onto nylon membrane, UV-crosslinked and hybridized with a uromodulin 5'-probe. The bands reacting with the probe will then be subjected to subcloning.

The plasmid pBluescript (Stratagene, LaJolla, CA), which was used as the cloning vector, is to be restriction-digested using PstI, ApaI and SacI, respectively, phosphatase-treated, and the linearized pBluescript cloning vectors will be mixed with the correspondingly digested inserts, ligation buffer, T4 DNA ligase, and incubated at 16°C for 16 hours. Half of this ligation mixture will be used to transform $CaCl_2$-prepared competent JM109 bacterial cells and then screened using small-scale plasmid preparations, which are carried out using mini-prep columns (Promega) and then restriction-digested to release the inserts. Through these procedures, the DNA fragments containing mouse uromodulin promoter are to be subcloned.

Detailed Restriction Mapping of Mouse Uromodulin Promoter

Restriction mapping of the 5'-flanking sequence of uromodulin, an important step for determining the restriction fragments for constructing transgenes has been performed. Although the detailed restriction map is not shown here, such a restriction map can be generated quite readily using any of the numerous publicly or commercially available DNA analysis software programs. A schematic presentation of the mouse uromodulin promoter with several restriction sites denoted is shown in Fig. 11.

EXAMPLE 2: ISOLATION OF GOAT UROMODULIN GENE PROMOTER

Isolation of Goat Uromodulin cDNA

The goat uromodulin cDNA was isolated using reverse transcriptase/polymerase chain reaction (RT-PCR) approach (Wu, et al., 1993). Briefly, a sense and an antisense primer were synthesized based on the mouse uromodulin gene sequence that was isolated in the laboratory of the present inventors. The sequences of these two primers are:

5'-GACTGAGTACTGGCGCAGCACAG-3' (SEQ ID NO:28) and

5'-GATTGCACTCAGGGGGCTCTGT-3' (SEQ ID NO:29). Total RNA was isolated from goat kidneys using the guanidine isothiocyanate method, reverse-transcribed using AMV reverse transcriptase, and the second strand of cDNA was synthesized using DNA polymerase I. PCR amplification was performed using total kidney cDNAs as templates and the two mouse uromodulin as primers, in the presence of dNTP, Taq polymerase, and PCR buffer. The PCR reaction was performed for 35 cycles of denaturation at 94°C, annealing at 55°C and extension at 72°C and the resulting PCR products were resolved by agarose gel. The products having the predicted size were subcloned into the TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced.

RT-PCR of goat kidney-derived mRNAs, using the pair of primers derived from mouse uromodulin, yielded a single, approximately 300 bp product upon agarose gel electrophoresis. The PCR product was subcloned and sequenced. A Blast search of Genbank of the PCR product sequence (SEQ ID NO:2; Fig.12) showed that the top four hits were uromodulin sequences from several species. Thus, the sequence of the PCR product shared

a 96% identity (287 bp/297 bp) with bovine uromodulin, 90% identity (218/241) with human uromodulin, a 78% identity (239/304) with rat uromodulin, and an 80% identity in a shorter stretch (125/156) with mouse uromodulin. The high degree of sequence identity of the PCR product with known uromodulin sequences firmly established that the product is a partial goat uromodulin cDNA.

Isolation of Goat Uromodulin Genomic DNA By Genomic Walking, Cloning and Sequencing

A genomic walking approach was employed to isolate the goat uromodulin gene using specific sequence information obtained from goat uromodulin cDNA. Genomic DNA was isolated from goat kidneys and used as templates for PCR-based genomic walking (Clontech, Palo Alto, CA). The genomic DNA was digested using five restriction enzymes (DraI, ScaI, EcoRV, PvuII, StuI), each of which created a blunt end in the genomic DNAs. The ends were ligated with adaptors. PCR was then performed using the ligated DNA library as templates, and two independent anti-sense primers synthesized based on the newly obtained uromodulin cDNA sequence as well as a sense primer located on the adaptor. The sequences for the two anti-sense primers are 5'-GTACCAGCCGCCCAGACTGACATCACAG-3' (SEQ ID NO:30; primer AS14), and 5'-CAGGTTGTACACGTAGTAGCCGCCGGCA-3' (SEQ ID NO:31; primer AS17). The PCR was performed for 1 cycle of denaturation at 99°C for 5 sec, annealing and extension at 68°C for 4 min., followed by 7 cycles of denaturation at 94°C for 2 sec, annealing and extension at 68°C for 4 min., followed by 32 cycles of denaturation at 94°C for 2 sec,

annealing and extension at 63°C for 4 min., and followed by 1 cycle at 63°C for 4 min. After the first round of PCR, the products were used as templates and subjected to a second round of PCR amplification using two new, nesting sense and anti-sense primers. The specific products were subcloned into the TA cloning vector and the identity of the goat uromodulin gene was confirmed by DNA sequencing of both ends of the product.

Based on the newly identified goat uromodulin cDNA, the two above anti-sense primers were designed for genomic walking using goat genomic DNA to identify DNA sequences that are located in the upstream region. After the first and second rounds of PCR and nesting PCR amplifications, a 1.5 kb, single PCR product was obtained. Subcloning and sequencing of this product revealed that its 3'-end shares 94% identity (494/522) with bovine uromodulin cDNA sequence, thus confirming that the PCR product is a portion of the goat uromodulin gene. The 5'-sequence did not share any significant homology with any of the known uromodulin cDNA sequences and therefore most likely represents intron sequences. Based on the gene structure of mouse uromodulin and the relative length (1.5 kb) of the PCR product, this 5'-sequence is most likely located in intron 1. The nucleotide sequences of intron 1 (SEQ ID NO:3) and exon 3 (SEQ ID NO:4) of the goat uromodulin gene are shown in Figs. 13A and 13B, respectively.

Isolation of Goat Uromodulin Promoter by Secondary Genomic Walking

For the isolation of goat uromodulin promoter, the 5'-end of the genomic clone that was isolated from the first round of genomic walking was used to design new antisense "walking primers" located in intron 1. The five primers are:

5'-AAGATTTACCAGCCCGGGCCGTCGACC-3' (SEQ ID NO:32; AS1)

5'-AATAAAGTGCCAGGGCAGGGGGGCTTA-3' (SEQ ID NO:33; AS2)

5'-CTTGTGTGGTTGAGTGTGTTCTTGACC-3' (SEQ ID NO:34; AS3)

5'-TGTGAAAGGGGATGTCTTTGGGTACCA-3' (SEQ ID NO:35; AS4)

5'-ACAGCAATGTGCAACCCAATGGAAGGG-3' (SEQ ID NO:36; AS5).

Fresh goat genomic DNA as template was digested by the five blunt-ending restriction enzymes (see above) and subjected to PCR walking using these five anti-sense primers and the aforementioned conditions.

PCR and nesting PCR yielded a highly specific, 1.0 kb product in three independent primer combinations. A further round of genomic walking resulted in a 1.6 kb fragment which was subcloned as smaller fragments. Subcloning and DNA sequencing of the subcloned fragments provided the 1.6 kb goat uromodulin promoter sequence of SEQ ID NO:37 and its structural features as shown in Figs. 14A and 14B. A computer comparison/alignment of the nucleotide sequences of the mouse and goat uromodulin promoter regions is presented in Figs. 15A and 15B.

EXAMPLE 3: CONSTRUCTION OF KIDNEY-BASED BIOREACTOR SYSTEM

Construction of Chimeric Genes

To test the tissue-specificity of the uromodulin gene promoter and its utility in a kidney-based bioreactor system, a chimeric gene containing a uromodulin promoter and a gene encoding a pharmaceutically-important protein is to be constructed. For this purpose, human growth hormone (hGH), whose expression has been recently assessed in a uroplakin II-based, bladder bioreactor system (Kerr et al, 1998) will be tested first. A potential limitation has been recognized with the bladder bioreactor system in that it produced relatively low amounts of hGH. Such a potential limitation may possibly be associated with the less than optimal secretory activity of the urothelium. Since uromodulin is normally synthesized in the ascending limb of Henle's loop and the distal tubules where active secretion takes place, the present inventors expect that there will be an active secretion of synthesized hGH into the urine of mice, resulting in high protein yield. The presence of this uromodulin/hGH gene in transgenic mice will allow a comparison of the efficiency between the kidney-based and the bladder-based reactor systems.

An 8.8 kb genomic fragment containing the 5'-upstream region of the mouse uromodulin gene was used as a template for PCR amplification to yield a 3.0 kb uromodulin promoter fragment. PCR sense (SEQ ID NO:53) and antisense (SEQ ID NO:54) primers were designed so that their ends included an ApaI enzyme cleavage site to facilitate cloning. The 3.0 kb PCR fragment was subcloned into the ApaI site of

the pBluescript vector. A 2.1 kb genomic fragment of human growth hormone gene (Genbank accession number M13438 for complete coding sequence of hGH) containing the entire coding sequences was excised from phGH-N vector (obtained from Brian M. Shewchuk, Department of Genetics, University of Pennsylvania, Philadelphia, PA), gel-purified and subcloned into the BamHI site of the above-mentioned pBluescript vector so that human growth gene is positioned downstream of the mouse uromodulin promoter (Fig. 16). The correct orientation of the chimeric gene was verified by restriction digestion and DNA sequencing. The uromodulin-hGH chimeric gene was retrieved en bloc by restriction digestion using KpnI and XbaI. The 5.1 kb fragment was resolved by agarose gel electrophoresis, electroeluted and dialyzed extensively against Tris-EDTA buffer. The purified chimeric gene was then microinjected into the fertilized eggs of FVB/N inbred mice and implanted into the uteri of pseudopregnant mice as previously described by Brinster et al.(1981).

Southern blotting was employed to identify transgenic mice harboring the chimeric uromodulin-hGH gene. DNA was extracted from mouse tail using proteinase K digestion and NaCl precipitation. The DNA was digested with BglII, electrophoresed and transferred onto nylon membrane and hybridized with a 500 bp probe located at the 3'-end of the uromodulin promoter (Fig. 17). Out of 42 live-born animals, three carried the transgene as evidenced by the appearance of a 5.1 kb transgene fragment in the Southern blot of mouse tail DNA (Fig. 17). In Fig. 17, lanes 1 and 5 are non-transgenic

control mice showing the endogenous fragment (Endo) of uromodulin gene, transgene fragments (Trans), in mouse 2 (15 kb), mouse 3 (9 kb) and mouse 4 (5.5 kb and 4.9 kb). These different fragment sizes may reflect the particular transgene orientation and the chromosomal site of transgene integration.

Expression of hGH in Mouse Kidney

The expression of hGH in transgenic mouse kidney is to be assessed at both the mRNA and protein levels. RT-PCR will be performed to determine the expression of mRNA using primers specific for hGH. Total RNAs will be extracted from transgenic mouse kidneys and from control tissues, including rat liver, skin, intestine, stomach, brain, skeletal muscle, thymus, thyroid gland, bladder, lungs, heart, pancreas, spleen, prostate, seminal vesicles, uterus and ovaries. The total RNAs are to be reverse-transcribed, PCR amplified and analyzed by agarose gel electrophoresis. The results will reveal whether hGH is expressed in kidney-dependent fashion. To determine whether hGH was synthesized in the ascending limb of Henle's loop and the distal tubules of the kidney, immunofluorescent staining of the kidney using anti-hGH antibody will be performed. Frozen kidney sections are to be stained using an indirect immunofluorescent method (Wu et al, 1993).

The laboratory of the present inventors have now performed radioimmunoassays (RIA) to determine the level of hGH in the urine and the serum of the transgenic mice. Urine samples were collected from transgenic mice by gently

massaging the lower abdomen of the mice. Fresh samples were subjected to RIA without further processing. An RIA assay kit from Nichols Institute Diagnostics (San Juan Capistrano, CA) was used and ^{125}I-labeled hGH was obtained from Dupont NEN, (Billerica, MA). The standard curve was prepared by plotting the corrected CPM of each standard level against the standard concentration of hGH. The value of the urinary hGH concentration was obtained by referencing the CPM reading of the urine samples. For serum hGH measurement, whole blood was obtained from mouse tails and serum was isolated and subjected to RIA as described above.

Figure 18 shows the results from urine samples of two transgene-negative (-) and three transgene-positive mice (NOs. 1, 7, and 8) subjected to RIA. Human growth hormone was detected in transgenic mice Nos. 7 and 8, but not in transgenic mouse No. 1 or in the transgene-negative mice. The concentration of hGH in the two positive Nos. 7 and 8 mice were 20 and 22 ng/ml, respectively.

The urine and serum concentration of hGH in transgenic mice were also compared by RIA. Figure 19 shows the results of RIA performed on serum and urine samples from transgenic mice. The high concentration of hGH in transgenic mouse No. 8 (15 ng/ml) indicates leakage into the blood of hGH synthesized by kidney epithelial cells.

In order to assess the sensitivity of the RIA assay for hGH, known amounts of hGH were added into the same volume of urine sample from non-transgenic (normal) mice and then subjected to RIA. From Fig. 20, the recovery of hGH by this

assay is observed to be nearly 100% for hGH amounting to 10 ng/ml with the recovery being observed to decrease to 90% and 60%, respectively, when the hGH concentration increases to 20 to 50 ng/ml.

Having now fully described this invention, it will be appreciated that by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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